Effects of UV B and UV C radiation on viability, growth, and major natural compounds of *Malve neglecta* L. cells

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Abstract

Callus cultures were established *in vitro* from leaf explants of *Malva neglecta* and were sub-cultured several times until a rapid-growing cell line was obtained. The calluses were exposed to different doses of ultraviolet (UV) radiation as follows: 0, 432, 864, and 1296 J/m^2 for UV B and 0, 612, 1284, and 1836 J/m^2 for UV C, corresponding to 0, 30, 60, and 90 min radiations, in tandem. Exposure to UV, in particular for longer periods, reduced the viability and membrane integrity of Malva cells. This however, increased total contents of flavonoids, anthocyanins, and wall-bound phenolic acids. Among different pharmaceutical compounds of Malva cells increase of malvidin, catchin, cinnamic acid, and tannic acid was remarkable in UV-treated cells, compared to those of the control cells. According to the results, treatment of Malva cells with UV provides an attractive alternative to whole plants for effective production of specific phenolic compounds.

Keywords: Malva neglecta; Apigenin; Catechin; Delphinidin; Malvidin; Ultraviolet

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Introduction

The spectrum of ultraviolet (UV) reaching the Earth's surface has been divided into lower energy UV A (320-400 nm), higher energy UV B (280-320 nm), and UV C (254-280 nm) regions (Klose et al., 1987). The response of the plants to any given dose of radiation is species specific. Those parts of the ultraviolet daylight spectrum that particularly have attracted the most interests are UV-B (280-315 nm) and to less extent UV C bands (Barta et al., 2004). Ultraviolet radiation plays a key role in several biological functions, sometimes detrimental (e.g. DNA damage, immune suppression, cataracts) and others beneficial (e.g. assimilation of vitamin D, diminishing of risk of some internal cancers). However, there is no general health benefit in exposing crops and medicinal plants to extra UV B and UV C radiations (Zhang and Bjorn, 2009). It is well known that plants sense UV radiation in different ways although the molecular nature and cellular localization of the primary 'receptor' of the radiation is still unknown (Zhang and Bjorn, 2009; Brosche and Strid, 2003). Recent studies have shown that UV B affects a number of important physiological processes through different pathways including second messengers such as calcium, kinases and the catalytic formation of reactive oxygen species (ROS). High level of UV B causes cellular damage and oxidative stress, thus activating a general stress

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signal transduction pathway which leads to a response similar to the one which occurs after pathogen attack and other stresses (Ries et al., 2000). These responses include activation of ROS scavenging enzymes as well as production of flavonoids, anthocyanins and phenolic acids as effective antioxidants (Beggs et al., 1985; Matkowski, 2008; Ravindaran et al., 2001). Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease (Havsteen, 2002). Increase of antioxidants in crops as well as medicinal plants exposed to UV has been widely reported (Matkowski, 2008; Kumari et al., 2010; Nasibi and Kalantari, 2005). To the best of our knowledge however, few studies have considered UV radiation as a tool in biotechnology of production of pharmaceuticals by *in vitro*-cultured cells of medicinal plants.

Cheese plant (*Malva neglecta*, Malvaceae) is often considered a weed and is consumed as a food as well as a medicinal plant for treatment of skin, respiratory system diseases, and inflammation of the digestive or urinary systems (Gurbuz et al., 2005; Mavi et al., 2004). The plants also have important polyphenols e.g., apigenin, malvidin, delphinidin and catechin (Khare, 2007; Jayalakshmi et al., 2011; Dlar et al., 2012). The present study was undertaken to evaluate the effects of UV radiation on the production of pharmaceutical compounds in callus-cultured Malva cells.

Materials and Methods

Initiation of callus and treatment with UV

The calluses were established from leaves of *Malva neglecta* L. on solidified B5 basal media, supplemented with 22.53 mg/L of BA and 186.21 mg/L of NAA, pH 5.8 (Gamborg et al., 1968). The samples were surface sterilized by subsequent washing with detergent, sodium hypochlorite (containing 5% active chlorine, 20 min), ethanol (75%, 30 s) with rinsing in sterile distilled water intervals. After 7 days, the calluses were emerged, grown at 25 ± 2 °C in darkness and their media were renewed every 10 days. After frequent subcultures (11 times), when the cells were physiologically and genetically stabilized, they were exposed to UV B and UV C for three days, each day 0, 30, 60 and 90 min. Ultraviolet B and UV C were provided by appropriate lamps (Phillips TL/12 40 W, Netherlands, and Tuv/G30 T8, Phillips, Netherlands, respectively). The applied dosages were respectively equal to 0, 432, 864, and 1296 J/m^2 for UV B and 0, 612, 1284 and 1836 J/m^2 for UV C, and were decided according to other studies (Ghanati et al., 2007). The cells were then frozen in liquid nitrogen and stored at -80 °C for further biochemical analysis.

Evaluation of the growth and cell viability

Effect of UV on the growth of Malva cells was monitored by comparing fresh weight of treated cells with the control ones. Viability of the cells was assessed with Evans blue (Smith et al., 1984).

Biochemical analysis

Protein content was evaluated by the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford, 1976). Total content of soluble carbohydrates was measured by phenol-sulphuric acid method (Dubois et al., 1956). Anthocyanins were extracted from frozen cells with MeOH: HCl (99:1) and were measured at 550 nm (Masukasu et al., 2003). Flavonoids were extracted with acidified EtOH: HCl (99:1) and were measured by spectrophotometer at 270, 300 and 330 nm (Krizek et al., 1993). For detection of apigenin, catechin, malvidin and delphinidin the cells were homogenized in 8 mL of pure methanol followed by sonication (90 min) and centrifugation at 15000 rpm for 20 min. The supernatant of each sample was air-dried, re-dissolved in methanol and applied to HPLC. The compounds were eluted at a flow rate of 1 mL/min with a linear gradient of 30% - 100% acetonitrile and were detected at 340 nm, using commercially available authentic standards (Sigma, USA). Rate of membrane lipid peroxidation of Malva cells was determined by estimating malondialdehyde (MDA) content with thiobarbituric acid (TBA) and trichloroacetic acid (TCA). The content of wall-bound phenols was measured only in control cells and those which were exposed to UV B and UV C for 90 min. The

walls of these cells were separated by sequential washing with EtOH, $CHCl_3$-MeOH (2:1), and acetone. Phenolics were liberated from pulverized cell wall preparations with hot ammonium oxalate and NaOH, and then extracted with EtOAc before being analyzed by HPLC (Knauer, Germany). Phenolics were eluted from a C-18 column (Perfectsil Target ODS-3 (5µm), 250 × 4.6 mm, MZ-Analysentechnik, Mainz, Germany) using a linear gradient of 30% - 80% MeOH containing 0.1% AcOH at a flow rate of 0.5 ml/min, and were detected at 280 nm, using commercially available authentic standards (Sigma, USA) (Ghanati et al., 2005).

Data analysis

All experiments were repeated at least three times in triplicates. The SPSS (version 19) and Excel software were utilized for statistical analysis. LSD-test was calculated for multiple mean comparisons at a significance level of P ≤0.05.

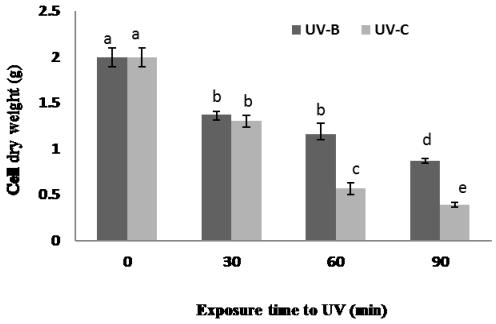

Fig. I. Effect of UV radiation on the growth of Malva cells. Data are means ± SD, n = 3. Signs with different letters in each group indicate significant differences at $P \leq 0.05$ according to LSD.

Results

Exposure to UV adversely affected the growth of Malva cells and this was more pronounced in UV C treatments (Fig. I). Similarly viability of the cells significantly decreased after exposure to UV B and UV C, compared to the control cells (Table 1). The most adverse effect of UV on cell viability was observed in prolonged

Table 1
Effects of UV on certain physiological parameters of Malva cells

Time (min)	UV B			UV C		
	Viability (%)	Protein (µg.mg FW^{-1})	Sugar	Viability (%)	Protein (µg.mg FW^{-1})	Sugar
0	100 ± 1^{a}	3.6 ± 0.3^{a}	96.9 ± 10^{a}	100 ± 1^{a}	3.6 ± 0.3^{a}	96.9 ± 9^{c}
30	68.7 ± 1^{b}	1.7 ± 0.1^{b}	111.9 ± 9^{a}	65.2 ± 3^{b}	1.6 ± 0.1^{b}	105.3 ± 9^{b}
60	58.04 ± 6^{b}	0.9 ± 0.1^{bc}	122.5 ± 9^{a}	28.3 ± 3^{cd}	1.1 ± 0.1^{c}	123.1± 14^{b}
90	43.5 ± 1^{c}	0.6 ± 0.05^{c}	126.4± 10^{a}	19.6 ± 0.1^{d}	0.9 ± 0.1^{c}	203.1 ±14^{a}

Different letters refer to significant differences at P≤0.05, according to LSD, n=3.

Table 2
Wall-bound phenolics of Malva cells before and after three days exposure to UV.

	Phenolic Acids (µg.g wall DW^{-1})					
	Gallic acid	Cinnamic acid	Ferulic acid	Tannic acid	Benzoic acid	Total
Control	0.7 ± 0.05	1.6 ± 0.7	17.5 ± 3	3.7 ± 0.3	4.2 ± 1	27.9 ± 3^{c}
UV B	7.03 ± 1	4.6 ± 1	26.5 ± 3	45.5 ± 12	27.3 ± 8	111.1 ± 20^{b}
UV C	35.5 ± 3	45.2 ± 1	69.3 ± 4	271.1± 39	131.1± 12	552.4 ± 46^{a}

Different letters refer to significant differences with control at P≤0.05, according to LSD, n=3.

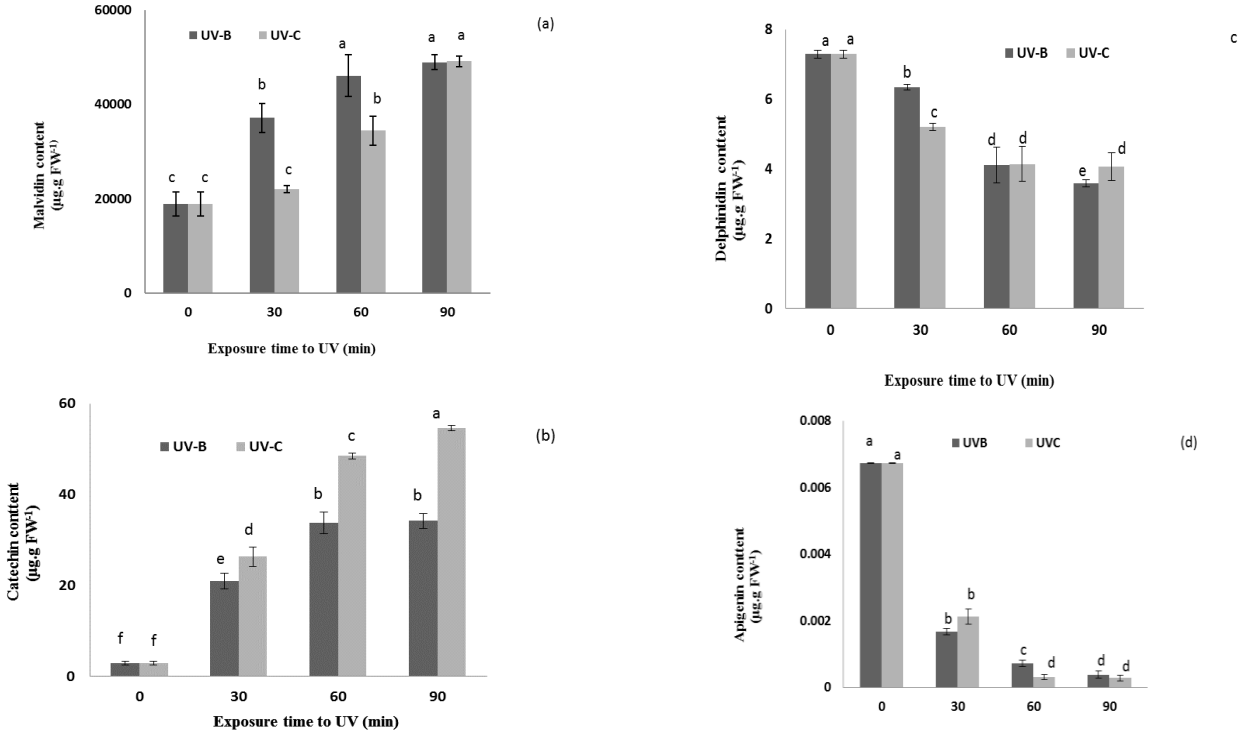

Fig. II. Major flavonoid compounds of Malva cells before and after exposure to UV B and UV C, a, malvidin; b, catechin; c,delphinidin;d, apigenin. Data are means ± SD, n = 3. Signs with different letters in each group indicate significant differences at $P \leq 0.05$ according to LSD.

exposure periods. Decrease of the viability of the cells was more pronounced under UV C treatments (Table 1). Protein contents of Malva cells decreased to 50% of the control after 30 min exposure to UV, and continuously decreased along with the increase of exposure period. There was no significant difference between the rate of decrease of protein contents of UV B and UV C treated cells (Table 1). Soluble sugar contents of UV-treated cells however, were significantly higher than those of the control cells (Table 1).

The content of wall-bound phenolics of Malva cells after 90 min UV radiation is shown in Table 2. Treatment of the cells with UV significantly increased total content of wall-bound phenolic compounds, compared to the control cells. The rate of increase of total wall-bound phenolics was much prominent in UV C than UV B treatment (Table 2). In both UV B- and UV C-treated cells increase of tannic acid and benzoic acid were more noticeable than other measured phenolics.

Treatment of Malva cells with UV B did not change anthocyanin contents, compared to the control cells (Table 3). Treatment with UV C for 60 and 90 min however, significantly

Table 3
Total contents of anthocyanins and flavonoids (UV-absorbent compounds) of Malva cells before and after exposure to UV.

Time (min)	UV B		UV C	
	Anthocyanins (µM.g FW^{-1})	flavonoids	Anthocyanins (µM.g FW-1)	flavonoids
0	199.2 ± 5^{a}	669.01 ± 56^{c}	199.2 ± 5^{b}	669.01 ± 56^{b}
30	215.5 ± 9^{a}	701.2 ± 39^{c}	215.7 ± 20^{b}	716.8 ± 29^{b}
60	223.03 ± 12^{a}	825.7 ± 32^{b}	253.6 ± 9^{a}	976.1 ± 47^{a}
90	227.8 ± 15^{a}	1273.9 ± 140^{a}	267.3 ± 20^{a}	1027.5 ± 160^{a}

Different letters refer to significant differences at p≤0.05, according to LSD, n=3.

increased anthocyanin contents of Malva cells, compared to the control ones (Table 3). Significant increase was observed in flavonoid contents of the cells after exposure to UV B and UV C for 60 and 90 min, in comparison with control cells (Table 3).

Among UV absorbing compounds, malvidin had the highest content and its content significantly increased after exposure of the cells to UV B, but only increased after longer periods of exposure to UV C, compared to those of the control cells (Fig. II a). In both UV B- and UV C-treated cells the content of catechin was significantly higher than those of the control cells (Fig. II b). The contents of delphinidin and apigenin of UV-treated cells however, were remarkably lower than those of the control cells (Figs. II c and II d, respectively).

In comparison with control condition, damage of membranes and production of MDA occurred in all periods of exposure to UV B, but in longer periods of exposure to UV C (Fig. III).

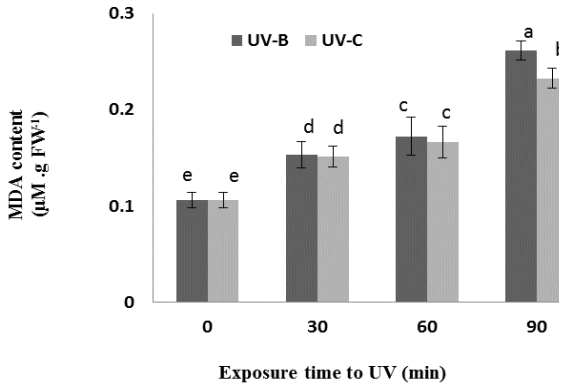

Fig. III. Membrane lipid peroxidation rate of Malva cells before and after exposure to UV B and UV C. Data are means ± SD, n = 3. Signs with different letters in each group indicate significant differences at $P \leq 0.05$ according to LSD.

Discussion

The viability of Malva cells and their protein content decreased after exposure to both kinds of UV B and UV C in the present study. Upon exposure of plant cells to UV, its energy reacts with nucleic acids and other vital cell components, resulting in injury or death of the exposed cells (Cohen et al., 1994). Ultraviolet radiation causes not only modification or destruction of amino acids, but leads to the inactivation of proteins and enzymes. The reason is the fact that the aromatic amino acids and disulfide groups strongly absorb UV radiation (Hollosy, 2002).

Soluble sugar contents of Malva cells increased after exposure to UV. Similar reports are available on the increase of soluble sugars after treatment with UV in other plants (Tegelberg et al., 2002). This may be resulted from altered carbon allocation or the breakdown of storage carbohydrates into soluble sugars. Moreover, the repair and UV-exclusion processes in UV-treated cells may have required soluble sugars rather than storage sugars (Tegelberg et al., 2002).

Ultraviolet exposure can induce oxidative stress in plant cells. Therefore, these cells normally muster several different defensive metabolic pathways on exposure to UV. Among defense mechanisms are reactive oxygen scavenging compounds and UV absorbing molecules such as flavonoid derivatives and their biosynthetic machineries (Brosche and Strid, 2003).

Increase of flavonoids, anthocyanins, tannins and wall-bound phenolics in UV-treated Malva cells might be a part of their defensive response. Mutants of *Arabidopsis* lacking flavonoids are hypersensitive to UV radiation whereas an *Arabidopsis* mutant possessing constitutive elevated accumulation of flavonoids and other phenolics is tolerant to lethal UV level (Bieza and Lois, 2001). Flavonoids strongly absorb light in the range of 220-380 nm and are known to be photo-stable (Stapleton, 1994). Malvidin was the main flavonoid compound of Malva cells followed by catechin, delphinidin, and apigenin. Increase of malvidin and delphinidin due to UV radiation has been observed in other species of Malva, *M. sylvestris* (Jayalakshmi et al., 2011). In the present study increase of malvidin was more outstanding in UV B treatments, and catechins remarkably increased in UV C treatments. According to the results presented here treatment of Malva cells with UV provides an attractive alternative to whole plants for effective production of specific phenolic compounds.

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